B3

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 :
A61K 35/00, 48/00, C12N 15/63, 15/85, 15/86, A01N 1/02

A1

(11) International Publication Number: WO 00/62787

(43) International Publication Date: 26 October 2000 (26.10.00)

(21) International Application Number: PCT/US00/09664

(22) International Filing Date: 11 April 2000 (11.04.00)

(30) Priority Data:
09/292,278 15 April 1999 (15.04.99) US

(71) Applicant: THE REGENTS OF THE UNIVERSITY OF CALIFORNIA [US/US]; 12th floor, 1111 Franklin Street, Oakland, CA 94067-5200 (US).

(72) Inventors: RAZ, Eyal; 13658 Mango Drive, Del Mar, CA 92014 (US). MARTIN-OROZCO, Elena; 3137 Via Alicante #C, La Jolla, CA 92037 (US).

(74) Agent: FRANCIS, Carol, L.; Bozicevic, Field & Francis LLP, Suite 200, 200 Middlefield Road, Menlo Park, CA 94025 (US).

(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: METHODS AND COMPOSITIONS FOR USE IN POTENTIATING ANTIGEN PRESENTATION BY ANTIGEN PRESENTING CELLS

(57) Abstract

The invention provides methods to enhance the antigen-presentation capabilities of cells capable of antigen presentation by contacting the cells with an immunostimulatory oligonucleotide. Through such contact, treated antigen presenting cells are induced to take up antigen (through upregulation of Fc R expression), present antigen (through upregulation of MHC Class I and II as well as CD1d expression), produce co-stimulatory factors (B7 and CD40), provide cell-to-cell adhesion (through upregulation of ICAM expression) and increase Th1 stimulatory cytokine production (e.g., IL-12), all at levels greater than are achieved through contact of the antigen presenting cells with antigen alone. Methods for use of the enhanced antigen presenting cells to activate T cells to respond to antigen are also provided, as are compositions of antigen presenting cells with activity enhanced according to the invention.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

METHODS AND COMPOSITIONS FOR USE IN POTENTIATING ANTIGEN PRESENTATION BY ANTIGEN PRESENTING CELLS

STATEMENT OF GOVERNMENT RIGHTS

This invention was made with Government support under Grant No. PO1 AI40682, awarded by the National Institutes of Health. The Government may have certain rights in this invention.

FIELD OF THE INVENTION

The invention relates to methods and oligonucleotide compositions for use in enhancing the antigen-presentation activity of cells which have antigen-presentation capabilities.

HISTORY OF THE RELATED ART

Cognate interactions between antigen-presenting cells (APCs) and lymphocytes play a crucial role in the primary activation of T cells to respond to antigen. These interactions are coordinated by upregulation of MHC, adhesion and co-stimulatory molecules on the surface of antigen-stimulated cells with antigen-presentation capabilities (e.g., dendritic cells, macrophages, B cells and tumor cells), as well as by contact with cytokines. Without the contribution of these varying factors, T cells can respond to antigen stimulation (especially by weakly immunogenic antigens) by developing tolerance rather than reactivity.

For example, tumor antigen vaccine administration can lead to T cell tolerance, thus minimizing the efficacy of the vaccine in countering tumor development. Such tolerance is believed to be the result, in part, of the absence of co-stimulatory signals (e.g., binding by T cells of B7 ligand on APCs). To avoid such tolerance induction, B7 transduced tumor cells are being studied for use as anti-cancer vaccines, with mixed success.

SUMMARY OF THE INVENTION

The invention provides methods for upregulating the expression of certain cell surface molecules on cells capable of antigen-presentation (e.g., B cells and macrophages; collectively, "APCs"), thereby enhancing the antigen-presenting activity of such cells, as compared to levels of expression observed in unstimulated and antigen-stimulated cells. The enhancement of antigen-presenting activity in such cells ("APC-activation" of the cells) is provided by contacting the cells with an immunostimulatory oligonucleotide (ISS) and can be achieved not only in cells pre-primed with ISS before contact with antigen, but also in cells contacted with ISS and antigen in combination. APC-activation of target cells in

turn enhances primary activation of T cells to react against, rather than develop tolerance toward, antigen.

Thus, according to one aspect of the invention, ISS enhance the capacity of affected macrophages to take up antigen (through upregulation of FcγR expression), present antigen (through upregulation of MHC Class I and II as well as CD1d expression), produce co-stimulatory factors (B7 and CD40), provide cell-to-cell adhesion (through upregulation of ICAM expression) and increase Th1 stimulatory cytokine production (e.g., IL-12).

In another aspect of the invention, expression of MHC Class I and II molecules, B7-2, CD40, ICAM, IFN-γR and IL-2R by B cells (splenic and naive peripheral cells) is increased by ISS contact.

In another aspect of the invention, APC-activation of target cells in turn enhances activation of T cells to respond to presented antigen. An especially useful application of this aspect of the invention is in cancer immunotherapy, wherein activation of tumor antigen reactive T cells is enhanced, thereby improving the prospects for T cell immunotherapy against tumor cells.

In yet another aspect of the invention, ISS are used to enhance the tumor antigen presenting capacity of tumor cells. *In vivo*, APC-activated tumor cells have a proportionally greater impact on stimulation of the host protective immune response. In addition, tumor cells treated with ISS *ex vivo* are useful as vaccines through reintroduction into a host.

The invention also provides cells and compositions based on such cells which have been APC-activated according to the invention, including macrophages, B cells, dendritic cells and tumor cells.

BRIEF DESCRIPTION OF DRAWINGS

Figure 1: *In vitro induction of cell surface molecules on mouse splenic B cells.*

1A and 1B: Spleen cells ($2x10^6$/well) from BALB/c mice were cultured with ISS-ODN (1 μg/ml), M-ODN (1 μg/ml), or media alone for 48 hours and then stained for B220 (PE) and activation markers (FITC) for FACS analysis. The histograms represent the FITC staining of gated PE^+ $B220^+$ B cells. The numbers on the right side of the histogram represent the percentage of positive cells in comparison to the media control. The numbers in the upper left represent the mean fluorescence, which was used to compare expression levels per cell for the markers that are expressed constitutively in > 95% of the cells. Data represent results from 4 independent experiments.

Figure 2: *In vivo induction of cell surface molecules on mouse splenic B cells.*
2A and 2B. BALB/c mice were injected i.p. with ISS-ODN (50 μg/ml), M-ODN (50 μg/ml), or saline. Mice were sacrificed on days 2, 7, and 21 after injection, the spleens were harvested, and the cells were stained for expression of B220 (PE) and the activation markers (FITC) for FACS analysis. The histograms represent the FITC staining of gated PE^+ B cells. The numbers in the upper left represent the mean fluorescence, which was used to compare expression levels per cell for the markers that are expressed constitutively in >95% of the cells. Data are representative of the results obtained from 3 different mice per group.

Figure 3: *Inhibition of B cell proliferation does not effect B cell surface molecule expression.* To prevent proliferative response, spleen cells were gamma irradiated (1500 rad) or MMC-treated (50μg/ml) and then incubated with ISS-ODN (1 μg/ml) plus BrdU (15 μg/ml). After 48 hours incubation, cells were stained for B220 (PE) and the activation markers (FITC) checked by FACS analysis. The histograms represent the FITC staining of gated PE^+ B cells. "Untr" represents untreated cells, MMC represents mitomycin-treated cells and "Irrad" represents irradiated cells. The numbers on the right side of the histogram represent the percentage of positive cells in comparison to the media control. The numbers in the upper left represent the mean fluorescence, which was used to compare expression levels per cell for the markers that are expressed constitutively in >95% of the cells. Data are represent values from 3 independent experiments.

Figure 4: *In vitro pUC19 activation of splenic B cell surface molecule expression.*
Spleen cells ($2x10^6$/well) were transfected (using DOTAP) with pUC19 (10 μg/ml) for 4 hours, then washed and incubated for additional 48 hours in culture media and then stained for B220 (PE) and activation markers (FITC) for FACS analysis. The histograms represent the FITC staining of gated PE^+ B cells. The numbers on the right side of the histogram represent the percentage of positive cells in comparison to the media control. The numbers in the upper left represent the mean fluorescence, which was used to compare expression levels per cell for the markers that are expressed constitutively in >95% of the cells. Data represent values from 3 different experiments.

Figure 5: *Bone marrow derived macrophage (BMDM) cytokine and surface molecule expression profiles induced by ISS.*
A. *Cytokine profile obtained from BMDM.* BMDM ($2x10^5$/ml) were cultured *in vitro* with ISS-ODN (1μg/ml), M-ODN (1 μg/ml), pUC19 (10μg/ml), LPS (5 μg/ml), or media alone. The supernatant

were collected after 48 hours. Cytokine concentrations (IL-6, IL-12, TNF-α and IFN-γ) were measured by ELISA. No detectable levels of TNF-α and IFN-γ were observed in this assay. Data represent mean±SE from 3 different experiments.

B. *Induction of cell surface molecules on BMDM.* BMDM ($2x10^5$/ml) were incubated *in vitro* with ISS-ODN (1μg/ml), M-ODN (1 μg/ml), pUC19 (10 μg/ml), LPS (5 μg/ml), or media alone for 48 hours in culture media and then stained for Mac 3 (PE) and activation markers (FITC) for FACS analysis. The histograms represent the FITC staining of gated PE^+ macrophages. The numbers on the right side of the histogram represent the percentage of positive cells in comparison to the media control. The numbers in the upper left represent the mean fluorescence, which was used to compare expression levels per cell for the markers that are expressed constitutively in >95% of the cells. Data represents results from one experiment of 3 performed.

Figure 6: *APCs pre-primed with ISS in vivo efficiently activate naive T cells in vitro.*

T cell receptor-ovalbumin encoding transgenic (TCR-OVA TG) mice were injected i.d. with ISS-ODN or M-ODN or saline. Naive T cells from TCR-OVA TG mice (10^5/well) were mixed with the same number of accessory cells (APCs) obtained from ISS-ODN, M-ODN or saline injected mice, and different concentrations of OVA for 4 days.

A. For T cell proliferation assay, the cultures were incubated with [^{3}H] thymidine (1 μCi per well) for 18 hours and cells were harvested and [^{3}H] thymidine incorporation was determined.

B. Levels of IFN-γ in the culture supernatants upon stimulation with different concentrations of OVA. IL-5 and IL-4 were not detected in this system. Data represents the mean±S.D of triplicates of one representative experiment of 3 which were performed.

Figure 7: *Effect of ISS-ODN pre-priming on the subsequent T cell response to ß-galactosidase in vivo.*

Mice were injected i.d. with ISS-ODN or M-ODN 3 days prior to ß-gal injection or co-injected with ISS-ODN/ß-gal, M-ODN/ß-gal or ß-gal alone.

A. IFN-γ release by ß-gal stimulated $CD4^+$ splenocytes at week 12 after a single ß-gal injection. No IL-5 or IL-4 were detected in this assay.

B. T cell proliferation assay at week 12 after a single ß-gal injection. The data represent mean±SE of 4 mice/group.

Figure 8: *Postulated mechanisms for the induction of a Th1 response by ISS-ODN or ISS enriched pDNA.*

A. ISS-ODN or ISS-pDNA trigger innate immunity to release a distinctive set of Th1 promoting cytokines (IFNs, IL-12, and IL-18) and up-regulate the expression of a distinctive profile of cell surface molecules on APCs (Fc receptors, MHC molecules, co-stimulatory molecules, adhesion molecules and cytokine receptors). These two responses are partially related (dotted lines).

B. Upon antigen encounter, the activated APCs interact with naive Th cells. This cognate interaction between ISS-activated APCs and naive Th cells in the ISS-induces cytokine milieu (i.e., IFNs, IL-12 and IL-18) biases the differentiation of naive Th cells toward a Th1 phenotype.

DETAILED DESCRIPTION OF THE INVENTION

1. *Activity of ISS-ODN APC Activators*

Cancer vaccines have been thought to have promise because, unlike most protective vaccines, they are therapeutic; i.e., they elicit immune responses against antigens (tumor antigens) in residence in the host on target cells. In cancer vaccination, a primary goal is to introduce a tumor antigen (one present on the target tumor cells) to cells with the capability to present antigen to T cells to induce T cell reactivity to the antigen. In all known pathways of primary T cell activation, the efficacy of activation is intimately tied to the density of peptide/MHC complexes displayed on APCs and the particular costimulatory molecules expressed by APCs. Problematically, however, peptide antigens recognized by T cell epitopes tend to be inefficiently presented, leading to T cells of relatively low antigen reactivity.

Thus, potentiation of APC-activation of cells to enhance their capacity to present antigen to T cells is directly correlative with an improvement in T cell reactivity to the presented antigen. This aspect of the invention has significant consequences for the efficacy of T cell-mediated immunotherapies, such as is practiced through use of anti-tumor vaccines.

Those of ordinary skill in the onocological art will be familiar with, or can readily ascertain, materials and methods for use in the construction of anti-tumor vaccines. According to the invention, an ISS-ODN is administered together with the anti-tumor vaccine to serve as an APC activator. The ISS-ODN may be co-administered with the vaccine, administered before vaccination or conjugated to a component of the vaccine (e.g., to the peptide tumor antigen).

Alternatively, the ISS-ODN may be used as an APC activator in conjunction with *ex vivo* cancer immunotherapy with tumor-antigen expressing cells treated with ISS. Those of ordinary skill in the onocological art will be familiar with, or can readily ascertain, materials and methods for use in the *ex vivo* preparation of antigen-expressing tumor cells for use in cancer immunotherapy. ISS are prepared and administered for use in such therapies as further described below.

In this respect, ISS-ODN administered according to the invention act as APC activators and modulate the host immune response to an antigen in ways which include: (1) potentiation of the capacity of affected bone-marrow derived cells (e.g., macrophages) to take up antigen (through upregulation of FcγR expression), present antigen (through upregulation of MHC Class I and II as well as CD1d expression), produce co-stimulatory factors (B7 and CD40), provide cell-to-cell adhesion (through upregulation of ICAM expression) and increase Th1 stimulatory cytokine production (e.g., IL-12); (2) potentiation of expression of MHC Class I and II molecules by B cells or autologous tumor cells; (3) potentiation of expression of B7-2, CD40, ICAM, IFN-γR and IL-2R by B cells (splenic and naive peripheral cells); and, (4) enhancement of primary activation of T cells to respond to presented antigen. An understanding of these phenomena is assisted by the following non-limiting definitions:

a. An "enhanced" T cell mediated immune response is one produced by an APC activator of the invention; i.e., more potent antigen-specific T cell activation and reactivity to an antigen than is induced by a control, generally consisting of the antigen administered alone or with a conventional adjuvant. As demonstrated by the data provided in the Examples, a six-fold (approximate) improvement in some aspects of T cell activation and antigen reactivity can be achieved according to the invention.

b. "Core" nucleotide sequence refers to the motif of at least six nucleotides, including at least one unmethylated CpG motif, which is present in the ISS-ODN of the invention. The relative position of each CpG sequence in ISS-ODN with immunostimulatory activity in certain mammalian species (e.g., rodents) is 5'-CG-3' (i.e., the C is in the 5' position with respect to the G in the 3' position). Many known ISS-ODN flank the CpG motif with at least two purine nucleotides (e.g., GA or AA) and at least two pyrimidine nucleotides (5'-Purine-Purine-[C]-[G]-Pyrimidine-Pyrimidine-3').

The Examples provide representative data evidencing APC-activation acheivable according to the invention. In summary, ISS-ODN enhance the APC function of target cells via the induction of different categories of cell surface molecules. The up-regulation of cell surface molecules on APCs

involved in antigen uptake (FcγR), antigen presentation (MHC class I, class II, and CD1d), costimulation (B7 and CD40), and cell-cell adhesion (ICAM-1) combined with cytokine production (TNF, IFNs and IL-12), provide the basis of the strong Th1 adjuvant properties and for the pre-priming effect observed for ISS-ODN (see, outline of proposed mechanism of action of ISS-ODN as APC activators shown in Figure 8A and B). These activities would be expected to suppress pathogen replication, enhance pathogen-derived antigen presentation, activate humoral and cell mediated specific immunity, and instruct the adaptive immune system to generate a Th1 response against an invading microbe.

In particular, the uptake of antigen is enhanced by ISS-ODN mediated up-regulation of the Fcγ receptors (CD16/32) while antigen presentation is enhanced by the up-regulation of classical MHC class I and class II molecules (Figures 1-2), as well as non-classical MHC molecules (CD1d; *in vivo*, Figure 2).

ISS-ODN also reduce the threshold number of cognate (cell-to-cell) interactions required for APC activation of lymphocytes by selectively enhancing the expression of ICAM-1 adhesion molecules (Figure 1-2). ICAM-1 binding to its ligand LFA-1 stabilizes interactions between APCs and lymphocytes. Furthermore, ISS-ODN up-regulate the expression of the costimulatory molecules B7 and CD40 on B cells and macrophages. These molecules play a major role in T cell priming, activation, and differentiation via interactions with their specific receptors, CD28 and CD40L, respectively, on naive T cells.

The up-regulation of cell surface molecules on B cells was observed for both splenic B cells (mainly memory cells, Figure 1) and peripheral blood B cells (mainly naive cells). The same pattern of expression was also observed for both *in vitro* (Figure 1) and *in vivo* (Figure 2) ISS-ODN stimulation. The levels of expression *in vivo* were maximal one week after i.p. injection of ISS-ODN and dropped back to the baseline levels after 21 days for most of the parameters evaluated, except for MHC class I, MHC class II, ICAM-1, and CD1d which displayed a sustained expression (Figure 2).

Gamma irradiation of B cells or their treatment with mitomycin C did not modify the expression of the various cell surface molecules described earlier, ruling out the known mitogenic properties of ISS-ODN on B cells as the cause for this differential expression. Neutralizing the cytokines induced by ISS-ODN stimulation resulted only in partial inhibition of the differential expression of some of the cell surface molecules on B cells, indicating their partial role in the observed expression profile.

ISS-ODN also stimulate another subset of APCs, monocyte/macrophages, to secrete the cytokines IL-6, IL-12 and TNFα (primary stimulation) (Figure 5A), and to amplify the expression of MHC class I, B7-1, CD40, FcγR-CD16/32 and ICAM-1 (Figure 5B). As was discussed for B cells, this array of cell surface molecules plays a major role in T cell activation and promotes T cell differentiation toward the Th1 phenotype.

The functional effects of ISS-ODN stimulated APCs on the subsequent T cell response was evaluated in different *in vivo* models: T cell proliferation in response to a model allergenic antigen (ovalbumin, or "OVA") in naive and ISS-ODN primed animals. In these models, ISS-ODN pre-priming enhanced the subsequent T cell proliferative response to OVA of naive OVA TCR TG T cells by 4 fold (Figure 6B). At the same time, OVA specific IFN-γ secretion was induced (*in vitro*), indicating the differentiation of naive Th cells (OVA TCR TG Th cells) toward a Th1 phenotype (Figure 6A). In this respect, the pre-priming of APCs with ISS-ODN instructs and biases the subsequent Th response to a Th1 type, emphasizing the instructive role of innate immunity (i.e., APCs) in the development of the adaptive immune response. This finding was reinforced in the second set of experiments in which ISS-ODN pre-priming was shown to be more efficient than ISS/antigen co-injection at stimulating the antigen-specific T cell proliferative response (2 fold) and $CD4^+$ T cell IFN-γ production (6 fold) (Figure 7).

2. *Structure and Preparation of ISS-ODN APC Activators*

Structurally, ISS-ODN are non-coding oligonucleotides having six or more nucleotides that may include at least one unmethylated CpG motif. The relative position of each CpG sequence in ISS-ODN with immunostimulatory activity in certain mammalian species (e.g., rodents) is 5'-CG-3' (i.e., the C is in the 5' position with respect to the G in the 3' position). Many known ISS-ODN flank the CpG motif with at least two purine nucleotides (e.g., GA or AA) and at least two pyrimidine nucleotides (5'-Purine-Purine-[C]-[G]-Pyrimidine-Pyrimidine-3'). CpG motif-containing ISS-ODN are believed to stimulate B lymphocyte proliferation (see, e.g., Krieg, *et al.*, *Nature*, 374:546-549, 1995).

The core hexamer structure of the foregoing ISS-ODN may be flanked upstream and/or downstream by any number or composition of nucleotides or nucleosides. However, ISS-ODN are at least 6 mer in length, and preferably are between 6 and 200 mer in length, to enhance uptake of the ISS-ODN into target tissues. Those of ordinary skill in the art will be familiar with, or can readily identify,

reported nucleotide sequences of known ISS-ODN. For ease of reference in this regard, the following sources are especially helpful:

Yamamoto, *et al., Microbiol.Immunol.*, 36:983 (1992)
Ballas, *et al., J.Immunol.*, 157:1840 (1996)
Klinman, *et al., J.Immunol.*, 158:3635 (1997)
Sato, *et al., Science*, 273:352 (1996)

Each of these articles are incorporated herein by reference for the purpose of illustrating the level of knowledge in the art concerning the nucleotide composition of ISS-ODN.

In particular, ISS-ODN useful in the invention include those which have the following hexameric nucleotide sequences:

1. ISS-ODN having "CpG" dinucleotides; and,
2. Inosine and/or uracil substitutions for nucleotides in the foregoing hexamer sequences for use as RNA ISS-ODN.

For example, DNA based ISS-ODN useful in the invention include those which have the following hexameric nucleotide sequences:

AACGTT, AGCGTC, GACGTT, GGCGTT, AACGTC, AGCGTC, GACGTC, GGCGTC, AACGCC, AGCGCC, GACGCC, GGCGCC, AGCGCT, GACGCT, GGCGCT, TTCGAA, GGCGTT and AACGCC (respectively, SEQ.ID.Nos. 1-18).

ISS-ODN may be single-stranded or double-stranded DNA, single or double-stranded RNA and/or oligonucleosides. The ISS-ODN may or may not include palindromic regions. If present, a palindrome may extend only to a CpG motif, if present, in the core hexamer sequence, or may encompass more of the hexamer sequence as well as flanking nucleotide sequences.

The nucleotide bases of the ISS-ODN which flank the CpG motif of the core hexamer and/or make up the flanking nucleotide sequences may be any known naturally occurring bases or synthetic non-natural bases (e.g., TCAG or, in RNA, UACGI). Oligonucleosides may be incorporated into the internal region and/or termini of the ISS-ODN using conventional techniques for use as attachment

points for other compounds (e.g., peptides). The base(s), sugar moiety, phosphate groups and termini of the ISS-ODN may also be modified in any manner known to those of ordinary skill in the art to construct an ISS-ODN having properties desired in addition to the described activity of the ISS-ODN. For example, sugar moieties may be attached to nucleotide bases of ISS-ODN in any steric configuration.

In addition, backbone phosphate group modifications (e.g., methylphosphonate, phosphorothioate, phosphoroamidate and phosphorodithioate internucleotide linkages) can confer anti-microbial activity on the ISS-ODN and enhance their stability *in vivo*, making them particularly useful in therapeutic applications. A particularly useful phosphate group modification is the conversion to the phosphorothioate or phosphorodithioate forms of the ISS-ODN oligonucleotides. In addition to their potentially anti-microbial properties, phosphorothioates and phosphorodithioates are more resistant to degradation *in vivo* than their unmodified oligonucleotide counterparts, making the ISS-ODN of the invention more available to the host.

3. *Synthesis of, and Screening for, ISS-ODN*

ISS-ODN can be synthesized using techniques and nucleic acid synthesis equipment which are well-known in the art. For reference in this regard, see, e.g., Ausubel, *et al., Current Protocols in Molecular Biology,* Chs. 2 and 4 (Wiley Interscience, 1989); Maniatis, *et al., Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., New York, 1982); U.S. Patent No. 4,458,066 and U.S. Patent No. 4,650,675. These references are incorporated herein by reference for the sole purpose of demonstrating knowledge in the art concerning production of synthetic oligonucleotides. Because the ISS-ODN is non-coding, there is no concern about maintaining an open reading frame during synthesis.

Alternatively, ISS-ODN or ISS enriched DNA may be isolated from microbial species (especially mycobacteria) using techniques well-known in the art, such as nucleic acid purification or hybridization. Preferably, such isolated ISS-ODN will be purified to a substantially pure state; i.e., to be free of endogenous contaminants, such as lipopolysaccharides. ISS-ODN isolated as part of a larger polynucleotide can be reduced to the desired length by techniques well known in the art, such as by endonuclease digestion. Those of ordinary skill in the art will be familiar with, or can readily ascertain, techniques suitable for isolation, purification and digestion of polynucleotides to obtain ISS-ODN of potential use in the invention.

Confirmation that a particular oligonucleotide has the properties of an ISS-ODN useful in the invention can be obtained by evaluating whether the ISS-ODN affects cytokine secretion and IgG antibody isotype production as described in Section A.2(e), above. Details of *in vitro* techniques useful in making such an evaluation are given in the Examples; those of ordinary skill in the art will also know of, or can readily ascertain, other methods for measuring cytokine secretion and antibody production along the parameters taught herein.

For use in the methods of the invention, the ISS-ODN APC activators of the invention will take the form of free ISS-ODN oligonucleotides, ISS-ODN oligonucleotide-peptide conjugates and ISS-containing recombinant expression vectors (data regarding the activity of ISS-ODN conjugates and ISS-ODN vectors are set forth in co-pending, commonly assigned U.S. patent applications Serial Nos. 60/028,118 and 08/593,554; data from which is incorporated herein by reference to demonstrate ISS-ODN immunostimulatory activity *in vivo*). In a vaccine composition, antigen may be co-delivered (separately or in an admixture with free oligonucleotides), expressed recombinantly from a plasmid (especially one containing the ISS-ODN moiety in the backbone) or conjugated to an antigen.

Examples of other useful conjugate partners include any immunogenic antigen (including allergens, live and attenuated viral particles and tumor antigens), targeting peptides (such as receptor ligands, antibodies and antibody fragments, hormones and enzymes), non-peptidic antigens (coupled via a peptide linkage, such as lipids, polysaccharides, glycoproteins, gangliosides and the like) and cytokines (including interleukins, interferons, erythropoietin, tumor necrosis factor and colony stimulating factors). Such conjugate partners can be prepared according to conventional techniques (e.g., peptide synthesis) and many are commercially available.

Those of ordinary skill in the art will also be familiar with, or can readily determine, methods useful in preparing oligonucleotide-peptide conjugates. Conjugation can be accomplished at either termini of the ISS-ODN or at a suitably modified base in an internal position (e.g., a cytosine or uracil). For reference, methods for conjugating oligonucleotides to proteins and to oligosaccharide moieties of Ig are known (see, e.g., O'Shannessy, *et al., J.Applied Biochem.,* 7:347 (1985), the disclosure of which is incorporated herein by reference solely to illustrate the standard level of knowledge in the art concerning oligonucleotide conjugation). Another useful reference is Kessler: "Nonradioactive Labeling Methods for Nucleic Acids", in Kricka (ed.), *Nonisotopic DNA Probe Techniques* (Acad.Press, 1992)).

Briefly, examples of known, suitable conjugation methods include: conjugation through 3' attachment via solid support chemistry (see, e.g., Haralambidis, *et al., Nuc.Acids Res.*, 18:493 (1990) and Haralambidis, *et al., Nuc.Acids Res.*, 18:501 (1990) [solid support synthesis of peptide partner]; Zuckermann, *et al., Nuc.Acids Res.*, 15:5305 (1987), Corey, *et al., Science,* 238:1401 (1987) and Nelson, *et al., Nuc. Acids Res.*, 17:1781 (1989) [solid support synthesis of oligonucleotide partner]). Amino-amino group linkages may be performed as described in Benoit, *et al., Neuromethods*, 6:43 (1987), while thiol-carboxyl group linkages may be performed as described in Sinah, *et al., Oligonucleotide Analogues:A Practical Approach* (IRL Press, 1991). In these latter methods, the oligonucleotide partner is synthesized on a solid support and a linking group comprising a protected amine, thiol or carboxyl group opposite a phosphoramidite is covalently attached to the 5'-hydroxyl (see, e.g., U.S. Patent Nos. 4,849,513; 5,015,733; 5,118,800 and 5,118,802).

Linkage of the oligonucleotide partner to a peptide may also be made via incorporation of a linker arm (e.g., amine or carboxyl group) to a modified cytosine or uracil base (see, e.g., Ruth, *4th Annual Congress for Recombinant DNA Research* at 123). Affinity linkages (e.g., biotin-streptavidin) may also be used (see, e.g., Roget, *et al., Nuc.Acids Res.*, 17:7643 (1989)).

Methods for linking oligonucleotides to lipids are also known and include synthesis of oligo-phospholipid conjugates (see, e.g., Yanagawa, *et al., Nuc. Acids Symp.Ser.*, 19:189 (1988)), synthesis of oligo-fatty acids conjugates (see, e.g., Grabarek, *et al., Anal.Biochem.*, 185:131 (1990)) and oligo-sterol conjugates (see, e.g., Boujrad, *et al., Proc.Natl.Acad.Sci USA*, 90:5728 (1993)).

Each of the foregoing references is incorporated herein by reference for the sole purpose of illustrating the level of knowledge and skill in the art with respect to oligonucleotide conjugation methods.

Co-administration of a peptide drug with an ISS-ODN according to the invention may also be achieved by incorporating the ISS-ODN in *cis* or in *trans* into a recombinant expression vector (plasmid, cosmid, virus or retrovirus) which codes for any therapeutically beneficial protein deliverable by a recombinant expression vector. If incorporation of an ISS-ODN into an expression vector for use in practicing the invention is desired, such incorporation may be accomplished using conventional techniques which do not require detailed explanation to one of ordinary skill in the art.

For review, however, those of ordinary skill may wish to consult Ausubel, *Current Protocols in Molecular Biology*, *supra*.

Briefly, construction of recombinant expression vectors (including those which do not code for any protein and are used as carriers for ISS-ODN) employs standard ligation techniques. For analysis to confirm correct sequences in vectors constructed, the ligation mixtures may be used to transform a host cell and successful transformants selected by antibiotic resistance where appropriate. Vectors from the transformants are prepared, analyzed by restriction and/or sequenced by, for example, the method of Messing, *et al., (Nucleic Acids Res.*, 9:309, 1981), the method of Maxam, *et al., (Methods in Enzymology*, 65:499, 1980), or other suitable methods which will be known to those skilled in the art. Size separation of cleaved fragments is performed using conventional gel electrophoresis as described, for example, by Maniatis, *et al., (Molecular Cloning*, pp. 133-134, 1982).

Host cells may be transformed with expression vectors and cultured in conventional nutrient media modified as is appropriate for inducing promoters, selecting transformants or amplifying genes. The culture conditions, such as temperature, pH and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan. If a recombinant expression vector is utilized as a carrier for the ISS-ODN of the invention, plasmids and cosmids are particularly preferred for their lack of pathogenicity. However, plasmids and cosmids are subject to degradation *in vivo* more quickly than viruses. Alternatively, viral vectors that can be utilized in the invention include adenovirus, adeno-associated virus, herpes virus, vaccinia or an RNA virus such as a retrovirus. Of the viral vector alternatives, adeno-associated viruses would possess the advantage of low pathogenicity. The relatively low capacity of adeno-associated viruses for insertion of foreign genes would pose no problem in this context due to the relatively small size in which ISS-ODN of the invention can be synthesized.

If modification of the phosphate group of an ISS-ODN is desired (e.g., to increase its bioavailability), the techniques for making phosphate group modifications to oligonucleotides are known in the art and do not require detailed explanation. For review of one such useful technique, the intermediate phosphate triester for the target oligonucleotide product is prepared and oxidized to the naturally occurring phosphate triester with aqueous iodine or with other agents, such as anhydrous amines. The resulting oligonucleotide phosphoramidates can be treated with sulfur to yield phophorothioates. The same general technique (excepting the sulfur treatment step) can be applied to yield methylphosphoamidites from methylphosphonates. For more details concerning phosphate group

modification techniques, those of ordinary skill in the art may wish to consult U.S. Patent Nos. 4,425,732; 4,458,066; 5,218,103 and 5,453,496, as well as *Tetrahedron Lett.* at 21:4149 (1995), 7:5575 (1986), 25:1437 (1984) and *Journal Am.ChemSoc.*, 93:6657 (1987), the disclosures of which are incorporated herein for the sole purpose of illustrating the standard level of knowledge in the art concerning preparation of these compounds.

C. *Pharmaceutical Compositions of ISS-ODN APC Activators*

ISS-ODN may be prepared in a pharmaceutically acceptable composition. Pharmaceutically acceptable carriers preferred for use with the ISS-ODN of the invention may include sterile aqueous of non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/ aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers (such as those based on Ringer's dextrose), and the like. Preservatives and other additives may also be present such as, for example, antimicrobials, antioxidants, chelating agents, and inert gases and the like. A composition of ISS-ODN may also be lyophilized using means well known in the art, for subsequent reconstitution and use according to the invention.

Absorption promoters, detergents and chemical irritants (e.g., keritinolytic agents) can enhance transmission of an ISS-ODN composition into a target tissue. For reference concerning general principles regarding absorption promoters and detergents which have been used with success in mucosal delivery of organic and peptide-based drugs, see Chien, *Novel Drug Delivery Systems*, Ch. 4 (Marcel Dekker, 1992).

Examples of suitable nasal absorption promoters in particular are set forth at Chien, *supra* at Ch. 5, Tables 2 and 3; milder agents are preferred. Suitable agents for use in the method of this invention for mucosal/nasal delivery are also described in Chang, *et al., Nasal Drug Delivery*, "Treatise on Controlled Drug Delivery", Ch. 9 and Table 3-4B thereof, (Marcel Dekker, 1992). Suitable agents which are known to enhance absorption of drugs through skin are described in Sloan, Use of Solubility Parameters from Regular Solution Theory to Describe Partitioning-Driven Processes, Ch. 5, "Prodrugs: Topical and Ocular Drug Delivery" (Marcel Dekker, 1992), and at places elsewhere in

the text. All of these references are incorporated herein for the sole purpose of illustrating the level of knowledge and skill in the art concerning drug delivery techniques.

ISS-ODN may also be prepared as part of a drug delivery system, such as a liposome. Liposomes are artificial membrane vesicles which are useful as delivery vehicles *in vitro* and *in vivo*. It has been shown that large unilamellar vesicles (LUV), which range in size from 0.2-4.0 μm can encapsulate a substantial percentage of an aqueous buffer containing large macromolecules. RNA, DNA and intact virions can be encapsulated within the aqueous interior and be delivered to cells in a biologically active form (Fraley, *et al., Trends Biochem. Sci.*, 6:77, 1981). In addition to mammalian cells, liposomes have been used for delivery of polynucleotides in plant, yeast and bacterial cells. In order for a liposome to be an efficient gene transfer vehicle, the following characteristics should be present: (1) encapsulation of the genes encoding the antisense polynucleotides at high efficiency while not compromising their biological activity; (2) preferential and substantial binding to a target cell in comparison to non-target cells; (3) delivery of the aqueous contents of the vesicle to the target cell cytoplasm at high efficiency; and (4) accurate and effective expression of genetic information (Mannino, *et al., Biotechniques*, 6:682, 1988).

The composition of the liposome is usually a combination of phospholipids, particularly high-phase-transition-temperature phospholipids, usually in combination with steroids, especially cholesterol. Other phospholipids or other lipids may also be used. The physical characteristics of liposomes depend on pH, ionic strength, and the presence of divalent cations.

Examples of lipids useful in liposome production include phosphatidyl compounds, such as phosphatidylglycerol, phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, sphingo-lipids, cerebrosides, and gangliosides. Particularly useful are diacylphosphatidylglycerols, where the lipid moiety contains from 14-18 carbon atoms, particularly from 16-18 carbon atoms, and is saturated. Illustrative phospholipids include egg phosphatidylcholine, dipalmitoylphosphatidylcholine and distearoylphosphatidylcholine.

The targeting of liposomes can be classified based on anatomical and mechanistic factors. Anatomical classification is based on the level of selectivity, for example, organ-specific, cell-specific, and organelle-specific. Mechanistic targeting can be distinguished based upon whether it is passive or active. Passive targeting utilizes the natural tendency of liposomes to distribute to cells of the reticulo-endothelial system (RES) in organs which contain sinusoidal capillaries. Active

targeting, on the other hand, involves alteration of the liposome by coupling the liposome to a specific ligand such as a monoclonal antibody, sugar, glycolipid, or protein, or by changing the composition or size of the liposome in order to achieve targeting to organs and cell types other than the naturally occurring sites of localization.

The surface of the targeted delivery system may be modified in a variety of ways. In the case of a liposomal targeted delivery system, lipid groups can be incorporated into the lipid bilayer of the liposome in order to maintain the targeting ligand in stable association with the liposomal bilayer. Various well known linking groups can be used for joining the lipid chains to the targeting ligand (see, e.g., Yanagawa, *et al., Nuc.Acids Symp.Ser.*, 19:189 (1988); Grabarek, *et al., Anal.Biochem.*, 185:131 (1990); Staros, *et al., Anal.Biochem.*, 156:220 (1986) and Boujrad, *et al., Proc.Natl.Acad.Sci.USA*, 90:5728 (1993), the disclosures of which are incorporated herein by reference solely to illustrate the standard level of knowledge in the art concerning conjugation of oligonucleotides to lipids). Targeted delivery of ISS-ODN can also be achieved by conjugation of the ISS-ODN to a the surface of viral and non-viral recombinant expression vectors, to an antigen or other ligand, to a monoclonal antibody or to any molecule which has the desired binding specificity.

D. *Methods and Routes for Administration of ISS-ODN APC Activators to a Host*

The ISS-ODN of the invention are administered to a host using any available method and route suitable for drug delivery, including *ex vivo* methods (e.g., delivery of cells incubated or transfected with an ISS-ODN) as well as systemic or localized routes. However, those of ordinary skill in the art will appreciate that methods and localized routes which direct the ISS-ODN into target tissue will be preferred in most circumstances to systemic routes of administration, both for immediacy of therapeutic effect and avoidance of oligonucleotide degradation *in vivo*.

Intranasal administration means are particularly useful in addressing respiratory conditions. Such means include inhalation of aerosol suspensions or insufflation of the polynucleotide compositions of the invention. Nebulizer devices suitable for delivery of ISS-ODN compositions to the nasal mucosa, trachea and bronchioli are well-known in the art and will therefore not be described in detail here. For general review in regard to intranasal drug delivery, those of ordinary skill in the art may wish to consult Chien, *Novel Drug Delivery Systems*, Ch. 5 (Marcel Dekker, 1992).

Dermal routes of administration, as well as subcutaneous injections, are useful in addressing conditions in the skin. Examples of means for delivering drugs to the skin are topical application of a

suitable pharmaceutical preparation, transdermal transmission, injection and epidermal administration.

For transdermal transmission, absorption promoters or iontophoresis are suitable methods. For review regarding such methods, those of ordinary skill in the art may wish to consult Chien, *supra* at Ch. 7. Iontophoretic transmission may be accomplished using commercially available "patches" which deliver their product continuously via electric pulses through unbroken skin for periods of several days or more. Use of this method allows for controlled transmission of pharmaceutical compositions in relatively great concentrations, permits infusion of combination drugs and allows for contemporaneous use of an absorption promoter.

Ophthalmic administration involves invasive or topical application of a pharmaceutical preparation to the eye. Eye drops, topical cremes and injectable liquids are all examples of suitable mileaus for delivering drugs to the eye.

Systemic administration involves invasive or systemically absorbed topical administration of pharmaceutical preparations. Topical applications as well as intravenous and intramuscular injections are examples of common means for systemic administration of drugs.

E. *Dosing Parameters for ISS-ODN APC Activators*

A particular advantage of the ISS-ODN of the invention is their capacity to exert APC activating activity even at relatively minute dosages. Although the dosage used will vary depending on the clinical goals to be achieved, a suitable dosage range is one which provides up to about 1-1000 μg of ISS-ODN/ml of carrier in a single dosage. In view of the teaching provided by this disclosure, those of ordinary skill in the clinical arts will be familiar with, or can readily ascertain, suitable parameters for administration of ISS-ODN according to the invention.

In this respect, it should be noted that the activity of ISS-ODN in the invention is essentially dose-dependent. Therefore, to increase ISS-ODN potency by a magnitude of two, each single dose is doubled in concentration. Clinically, it may be advisable to administer the ISS-ODN in a low dosage (e.g., about 1 μg/ml to about 50 μg/ml), then increase the dosage as needed to achieve the desired therapeutic goal. Alternatively, a target dosage of ISS-ODN can be considered to be about 1-10 μM in a sample of host blood drawn within the first 24-48 hours after administration of ISS-ODN. Based on current studies, ISS-ODN are believed to have little or no toxicity at these dosage levels.

Of course, all dosing decisions lie within the judgment of the clinician and, for applications where the ISS-ODN is administered as part of a vaccination protocol, must take into account the limits imposed by that protocol.

F. *Kits for Use in Practicing the Methods of the Invention*

For use in the methods described above, kits are also provided by the invention. Such kits may include any or all of the following: ISS-ODN (conjugated or unconjugated); a pharmaceutically acceptable carrier (may be pre-mixed with the ISS-ODN) or suspension base for reconstituting lyophilized ISS-ODN; additional medicaments; a sterile vial for each ISS-ODN and additional medicament, or a single vial for mixtures thereof; device(s) for use in delivering ISS-ODN to a host; assay reagents for detecting indicia that the APC activating effects sought have been achieved in treated animals and a suitable assay device.

Examples illustrating the practice of the invention are set forth below. The examples are for purposes of reference only and should not be construed to limit the invention, which is defined by the appended claims. All abbreviations and terms used in the examples have their expected and ordinary meaning unless otherwise specified.

EXAMPLE I

Experimental ISS-ODN APC-Activators

Endotoxin free (<1 ng/mg DNA) phosphorothioate single stranded ODNs (Trilink, San Diego, CA) were used in all the experiments described in these Examples. The sequence of the ISS-ODN was 5'-TGACTGTGAACGTTCGAGATGA-3'. An inactivated ISS-ODN ("M-ODN") was also synthesized for use as a control; its sequence was 5'-TGACTGTGAAGGTTAGAGATGA-3'. The underline indicates the ISS (CpG) motif and its corresponding alteration.

Plasmid pUC19 (which includes two copies of an AACGTT ISS-ODN core hexamer) was purified with the Qiagen MaxiPrep Kit (Chastworth CA). Activity was abolished in pUC19 by methylating the CpG moiety by incubating the plasmid with CpG methylase (M.*Sss* I, New England Biolabs, Beverly, MA) for 12 hours according to the manufacturer's instructions. The endotoxin level of the pUC19 plasmids was <1 ng/mg pDNA by the Pyrotel limulus amebocyte lysate (Assoc. Cape Cod, Woods Hole MA).

EXAMPLE II

ISS-ODN induce in vitro up-regulation of a distinctive profile of cell surface markers on splenic B cells

Female BALB/c mice and wild type (wt) control (C57B1/6) mice (six to ten weeks of age) were purchased from The Jackson Laboratories (Bar Harbor, ME). TCR-OVA (DO11.10) transgenic (TG) mice are produced on the BALB/c background to be responsive to a model antigen (ovalbumin, or "OVA") (David Broide, UCSD).

Splenic T cells and APCs were prepared from BALB/c TCR-OVA TG mice. The animals were injected i.d. with saline, ISS-ODN (50 μg/mouse) or M-ODN (50 μg/mouse) on days 0 and 7. On day 14 mice were sacrificed and the spleens were harvested as previosuly described (23) to obtain APCs. To enrich the APCs, splenocytes were treated with anti-CD8 abs (3.155) and anti-CD4 abs (RL172) followed by incubation with guinea pig complement (Pel-Freeze, Rogers, Arkansas) as described (24), and fixed with MMC (50 μg/ml, 30 min. at 37°C) prior to being used as accessory cells. Splenocytes were obtained from naive TCR-OVA TG mice and purified for T cells by negative selection using the following antibody cocktail: J11D (anti-HSA), CA4.12 (anti-Ia), RA36B2 (anti-B220), M5.114 (anti-Ia, isotype IgG2a), MAR.18 (mouse anti-Rat IgG) with guinea pig complement. The resultant T-cell preparations (>95% purity by FACS staining), 10^5/well, were mixed with the same number of accessory cells and hen egg ovalbumin (OVA, 10 μg/ml, grade 5, Sigma) for 3 or 4 days. The cultures were incubated with [^{3}H] thymidine (1 μCi per well) (ICN Pharmaceuticals Inc., Irvine, CA) for 18 hours. Cells were harvested and [^{3}H] thymidine incorporation was determined with a 1450 Microbeta liquid scintillation counter (Wallac, Turku, Finland).

Incubation of murine splenocytes with ISS-ODN for 48 hours resulted in the up-regulation of a distinctive profile of cell surface markers on the B220$^+$ population (Figure 1). As determined by FACS analysis, incubation with ISS clearly enhanced the expression of MHC class I, MHC class II, B7-2, CD40, ICAM-1, CD16/32, IFN-γR and IL-2R. A slight up-regulation was observed for B7-1. In contrast, CD23 expression was down-regulated, while no differences in the expression of CD49b, CD1d (Figure 1), CD49a, CD49c-f, IL-1R, and IL-6 R (data not shown) were observed in the ISS-ODN vs. Media or M-ODN treated cells. The cell surface profile induced by ISS stimulation of purified B splenocytes was similar to that observed for B cells (B220$^+$) from ISS stimulated splenocytes.

In contrast, stimulation with LPS resulted in a different pattern of activation, e.g., a stronger induction of B7-1 with less of an effect on CD23. The effect of LPS on other cell surface markers was similar to that observed for ISS-ODN but with different intensity (Figure 1).

To evaluate whether ISS-ODN could also activate resting B cells obtained from peripheral blood, one hundred BALB/c mice were bled. The lymphocytic population was purified by ficoll and cultured in media alone, with ISS-ODN, or with M-ODN for 48 hours, under the same conditions described above for splenocytes, and then stained and analyzed by FACS. ISS-ODN induced a similar activation profile on peripheral blood $B220^+$ cells as described for splenic $B220^+$ B cells *in vitro*.

EXAMPLE III

ISS-ODN induces in vivo up-regulation of cell surface markers on splenic B cells

In order to determine whether the B cell activation profile observed *in vitro* is also induced *in vivo*, BABL/c mice were injected with ISS-ODN, M-ODN (50 μg/50 μl saline/mouse) or saline i.p. Mice were sacrificed at days 2, 7, and 21 after the injection and the splenic $B220^+$ B cells were stained and analyzed by FACS.

The activation profile obtained *in vivo* was similar to that obtained *in vitro* (Example II), but the magnitude of expression was weaker. Additionally, CD1d was up-regulated *in vivo* (Figure 2) while it was unchanged *in vitro* (Figure 1). The differential expression of the cell surface molecules was detected by day 2 after ISS injection and reached its peak at day 7. At day 21 the differential expression for B7-1, CD40, CD16/32, as well as IL-2R and IFN-γR had waned, while the expression of class I, class II, CD1d and ICAM-1 molecules were still up-regulated (Figure 2).

EXAMPLE IV

ISS-ODN induces up-regulation of a distinctive profile of cells surface markers in vitro on bone-marrow-derived-macrophages (BMDM)

Bone marrow cells were flushed from the femurs of 4-to-8-week old female BALB/c mice, washed once with RPMI media and cultured in 145 mm petri dishes (Greiner Labortechnik, Kremsmunster, Austria). The culture medium consisted to EMEM (Irvine Scientific) supplemented with 10% FBS (Gemini Bioproducts Inc.), 2 mM L-glutamine (Bio-Whitaker), 1 mM sodium pyruvate (Bio-Whitaker), 30 ng recombinant murine M-CSF (R&D systems, Minneapolis, MN), 100 U penicillin/ml (Bio-Whitaker), 100 μg streptomycin (Bio-Whitaker), 0.125 mg/ml amphotericin B (Bio-Whitaker) and 30% L-cell conditioned media obtained by incubation of L929 cells with the media described above for 1 week. By 8 to 10 days of incubation, the cells had acquired

macrophage-like morphology. Cells then were washed and re-cultured with media containing ISS-ODN (1 μg/ml), M-ODN (1 μg/ml), LPS (5 μg/ml), pUC19 (10 μg/ml) or methyl-pUC19 (10 μg/ml). After 48 hours the cells were harvested by incubation with ice-cold PBS and prepared for either FACS analysis or a cytokine release assay.

After 8-10 days in culture, bone marrow derived macrophages (BMDM) were washed extensively and incubated with ISS-ODN, M-ODN, pUC19, methyl-pUC19 or LPS for 48 hours. Cytokine production was assessed by ELISA and the cell surface marker expression profile was analyzed by FACS. As shown in Figure 5A, ISS-ODN an dpUC19 but not M-ODN or methyl-pUC19 induced the production of IL-6 and IL-12 (IFN-γ was not induced and TNF-α was not detected at 48 hours). As shown in Figure 5B, expression of MHC class I, B7-1, CD40, CD16/32 and ICAM-1 on BMDM was also enhanced. In contrast to B cells, ISS-ODN or pUC19 did not modify the expression of MHC class II, B7-2, IL-2R or CD23 on BMDM, probably due to the lack of IFN-γ induction in this system.

Interestingly, based on the cytokine levels measured in the supernatants, BMDM derived from BALB/c mice secreted 17 fold more IL-6 and 43 fold more IL-12 than did BALB/c splenocytes upon stimulation with ISS-ODN, suggesting that these cells play a major role in the generation of the cytokine milieu induced by ISS-ODN-based DNAs *in vivo*.

EXAMPLE V

ISS-ODN enhancement of the functional ability of APCs to initiate an immune response in vivo

The data presented in the preceding examples demonstrate that, in addition to cytokine production and B cell proliferation, ISS-ODN up-regulates a distinct profile of cell surface molecules on various APCs (e.g., macrophages and B cells) involved in priming and shaping the subsequent T cell response (i.e., Th1 vs. Th2). Two different *in vivo* systems were used to evaluate the functionality of ISS-treated APCs (splenocytes) to activate T cells.

First, TCR-OVA TG mice were injected with ISS-ODN, M-ODN or saline. TCR-OVA TG mice with naive T cells (2 mice/group) were injected intradermally (i.d.) At the base of the tail with ISS-ODN (50 μg/mouse), M-ODN (50 μg/mouse), or saline on days 0 and 7. Mice were killed at day 14 for T cell proliferation and cytokine release assays. Twelve weeks after protein injection the mice were sacrificed. In the second set of experiments, BALB/c mice (4 mice/group) were injected i.d. (at the base of the tail) with ISS-ODN (50 μg/mouse) at the same site. As control groups, mice were co-injected i.d. at day 0 with either ISS-ODN (50 μg/mouse) or M-ODN (50 μg/mouse) plus ß-gal (10

μg/mouse) or with ß-gal alone (10 μg/mouse, at day 0). Mice were again sacrified twelve weeks after protein injection.

Spleen cells from these mice were used as APCs to activate naive TG OVA specific T cells in a T cell proliferation assay (thymidine uptake), in the presence of different concentrations of OVA protein. APCs from ISS-ODN injected mice induced 4 fold more T cell proliferation (at 30 μg/ml of OVA) than APCs obtained from saline or M-ODN treated mice (Figure 6A), indicating an enhanced capacity of APCs obtained from treated mice to stimulate naive T cells. The activation of naive TCR-OVA TG T cells by ISS-activated APCs in the presence of OVA resulted in IFN-γ production (Figure 6B), but not in IL-4 or IL-5 production, indicating the differentiation of naive Th cells toward Th1 phenotype *in vitro* by ISS primed APCs.

Interestingly, ISS-ODN delivery prior to antigen administration created both an appropriate cytokine milieu (Figure 2) and induced up-regulation of various surface molecules on APCs for a period of at least 3 weeks (Figure 2). As a result, T cell activation and Th1 differentiation responsive to a subsequently administered antigen was enhanced. More particularly, ISS-ODN or M-ODN were injected into BALB/c mice 3 days prior to antigen (ß-gal) administration (pre-priming) and the subsequent immune response was compared to ISS/ß-gal co-injection as well as to injection of ß-gal alone. ISS-ODN pre-priming resulted in enhanced T cell proliferation to ß-gal (2 fold over ISS/ß-gal co-injection; Figure 7B) and in enhanced ß-gal specific IFN-γ production (6 fold over ISS/ß-gal co-injection) (Figure 7A).

Both pre-priming or co-injection of ISS-ODN with ß-gal resulted in the development of higher anti-ß-gal IgG and IgG2a antibodies than ß-gal in saline. Thus, ISS pre-priming amplified the immune response in general and the Th1 response in particular to the subsequently injected antigen.

EXAMPLE VI

B cell surface molecules are induced by pUC19

To analyze the primary effect of pDNA on cytokine release we incubated BALB/c splenocytes with pUC19 or methyl-pUC19 and followed the levels of the cytokines in the supernatants. As shown in Figure 4, incubation of pUC19 induced a similar profile of cytokines to that induced by ISS-ODN whereas methyl-pUC19 did not.

To analyze the effect of pDNA on the cell surface profile, mouse splenocytes were incubated or transfected with pUC19 for 48 hours prior to antibody staining and analysis by FACS. More particularly, transfections of mouse splenocytes with pUC19 (10 μg/ml) and methyl-pUC19 (10 μg/ml) were performed with DOTAP (Boehringer Manheim). Mouse spleen cells were resuspended at $3x10^5$ cells/ml in teflon tubes and incubated with a mix of plasmid and DOTAP for 4 hours at 37 C, and 5% CO_2. Cell were washed twice and resuspended at $2x10^6$ cells/ml in culture media. After 48 hours of incubation, the supernatants were collected for cytokine ELISA and the cells harvested and stained for FACS analysis as described above. Additionally, FACS analysis of splenocytes incubated with pUC19 (10μg/ml) and methyl-pUC19 (10 μg/ml) was made under the same conditions mentioned above without DOTAP.

As shown in Figure 4, transfection with pUC19 induced a similar cell surface expression profile on B splenocytes as did incubation with ISS-ODN (Figure 1) while incubation with pUC19 resulted in a weak to marginal induction of cell surface molecules on these cells.

EXAMPLE VII

Induction of cell surface molecules on B cells is not dependent upon B cell proliferation

To evaluate whether the effect of ISS-ODN on the expression of surface molecules on B cells was related to its mitogenic effect on B cells, splenocytes were gamma irradiated or treated with MMC and then incubated with ISS-ODN or M-ODN. The expression of surface markers was analyzed 48 hours later. The inhibition of proliferation was confirmed by the lack of BrdU incorporation.

More particularly, to inhibit the mitogenic effect of ISS-ODN (11,12), murine spleen cells were gamma irradiated (1500 rad) or treated with MMC (50 μg/ml for 30 min. At 37°C). To check whether under these conditions ISS-ODNs still modify the expression of surface molecules on B cells, splenocytes were incubated with ISS-ODN (1 μg/ml), M-ODN (1 μg/ml), LPS (5 μg/ml) or media alone. After a 48 hour incubation, cells were stained for expression of B220 versus cell surface markers. Additionally, the cells were incubated with BrdU (15 μg/ml, Boehringer Mannheim, Indianapolis, IN) in order to check the inhibition of proliferation after the different treatments.

As shown in Figure 3, ISS-ODN up-regulates the expressioin of cell surface molecules despite the inhibition of the B cell proliferative response of ISS either by irradiation or by MMC treatment,

indicating that the observed expression pattern is not the consequence of the ISS induced B cell mitogenesis.

The invention having been fully described, modifications and extensions thereof may become apparent to those of ordinary skill in the art. All such modifications and extensions are intended to fall within the scope of the appended claims.

The invention claimed is:

1. A method for inducing activation of T cells to respond to an antigen, the method comprising contacting cells which are capable of antigen-presentation with an immunostimulatory oligonucleotide (ISS-ODN) to produce antigen presenting cells (APCs) with enhanced antigen-presentation capabilities, as compared to antigen-activated APCs; which APCs with enhanced antigen-presentation capabilities then present antigen to the T cells.

2. The method according to Claim 1 wherein the APCs with enhanced antigen-presenting capabilities are B cells.

3. The method according to Claim 1 wherein the APCs with enhanced antigen-presenting capabilities are bone-marrow derived macrophages.

4. The method according to Claim 1 wherein the APCs with enhanced antigen-presenting capabilities are tumor cells.

5. The method according to Claim 4 wherein the antigen is a tumor antigen.

6. The method according to Claim 1 wherein the APCs with enhanced antigen-presentation capabilities are produced *in vivo* by administering the ISS-ODN to a host.

7. The method according to Claim 1 wherein the APCs which have enhanced antigen-presentation capabilities are produced *in vitro*.

8. The method according to Claim 6 wherein the ISS-ODN is administered to the host concomittantly with the antigen.

9. The method according to Claim 6 wherein the ISS-ODN is administered to the host without the antigen.

10. The method according to Claim 7 wherein the T cells are activated through *in vitro* contact with the APCs which have enhanced antigen-presentation capabilities.

11. The method according to Claim 7 wherein the T cells are activated through *in vivo* contact with the APCs which have enhanced antigen-presentation capabilities.

12. A method for producing antigen presenting cells (APCs) with enhanced antigen-presentation capabilities, as compared to antigen-activated APCs, comprising contacting cells which are capable of antigen-presentation with an immunostimulatory oligonucleotide (ISS-ODN).

13. The method according to Claim 12 wherein the APCs with enhanced antigen-presenting capabilities are B cells.

14. The method according to Claim 12 wherein the APCs with enhanced antigen-presenting capabilities are bone-marrow derived macrophages.

15. The method according to Claim 12 wherein the APCs with enhanced antigen-presenting capabilities are tumor cells.

16. The method according to Claim 12 wherein the APCs with enhanced antigen-presentation capabilities are produced *in vivo* by administering the ISS-ODN to a host.

17. The method according to Claim 12 wherein the APCs which have enhanced antigen-presentation capabilities are produced *in vitro.*

18. The method according to Claim 16 wherein the ISS-ODN is administered to the host concomittantly with an antigen.

19. The method according to Claim 16 wherein the ISS-ODN is administered to the host without an antigen.

20. Antigen-presenting cells (APCs) with enhanced antigen-presentation capabilities produced by contacting the APCs with an ISS-ODN.

21. A composition comprising the cells of Claim 20 and a pharmaceutically acceptable carrier.

FIGURE 1A

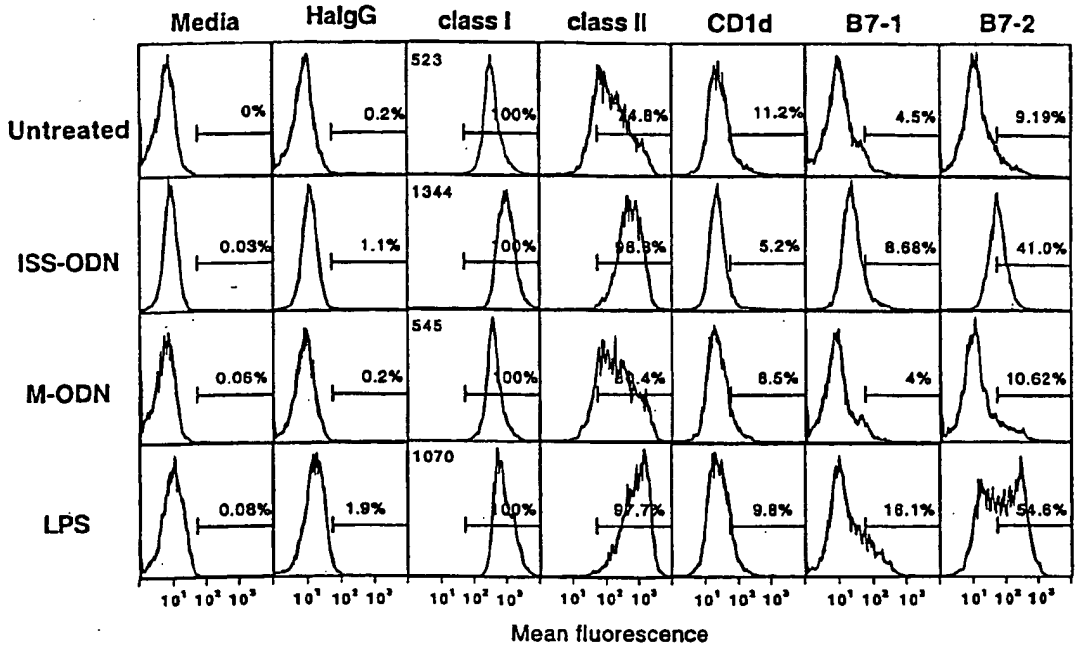


FIGURE 1B

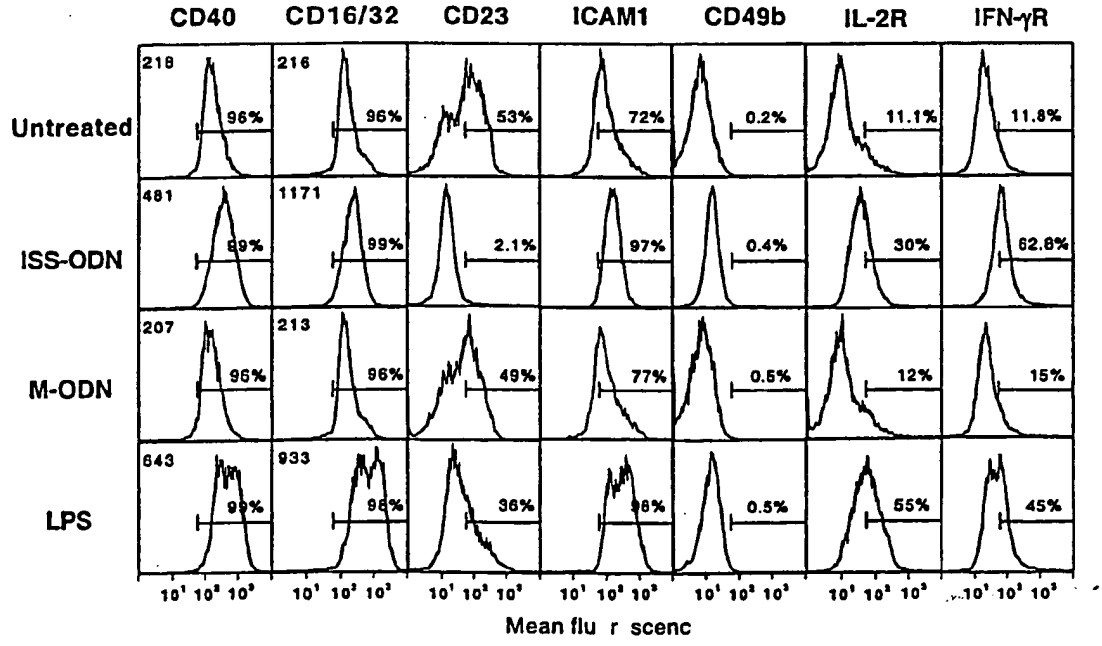


FIGURE 2A

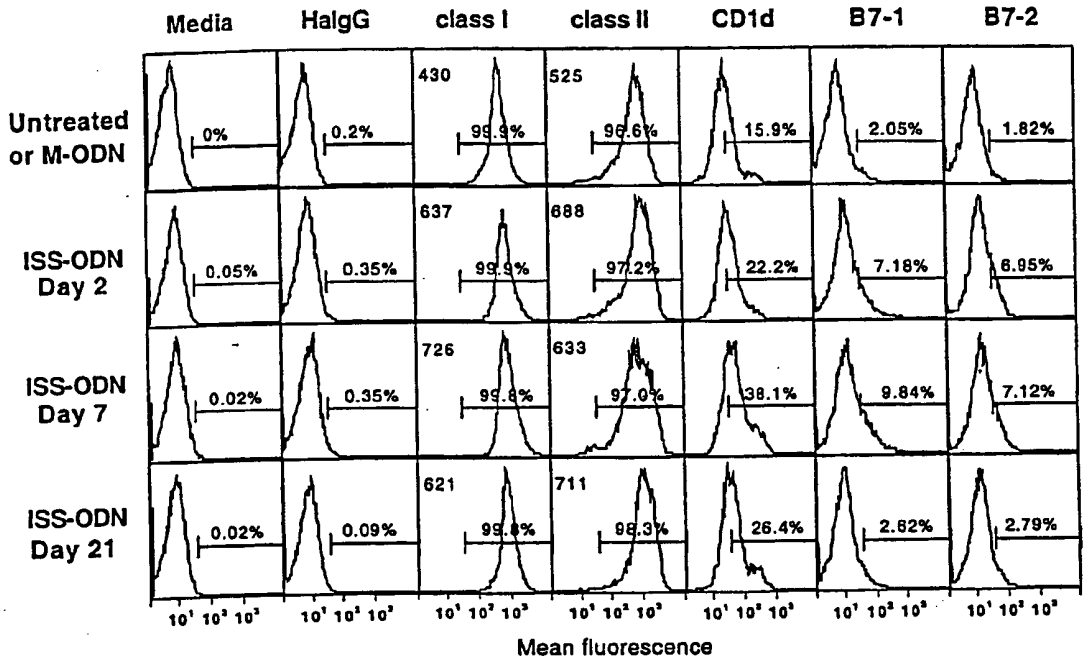


FIGURE 2B

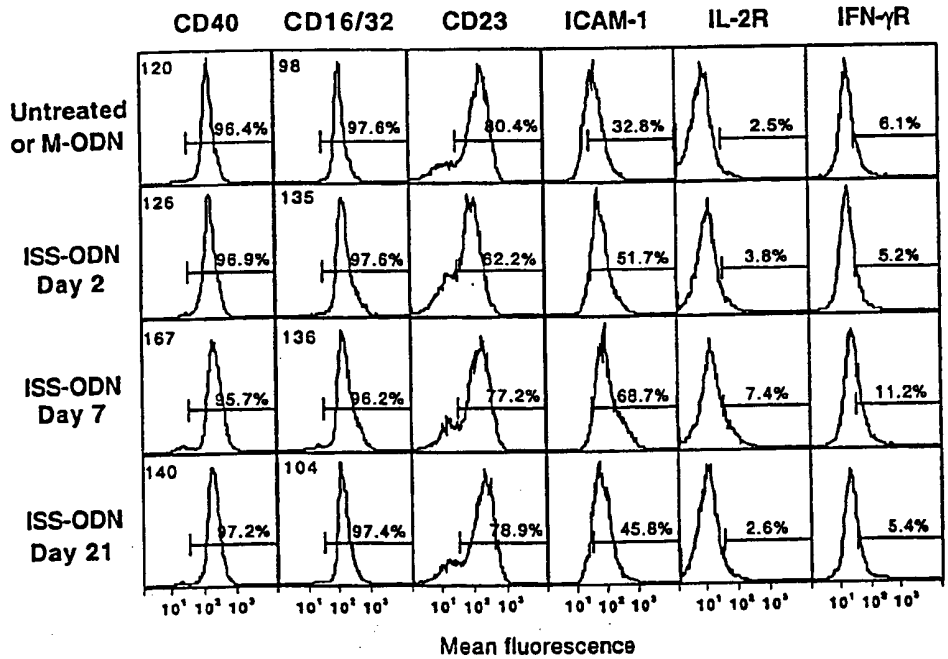


FIGURE 3

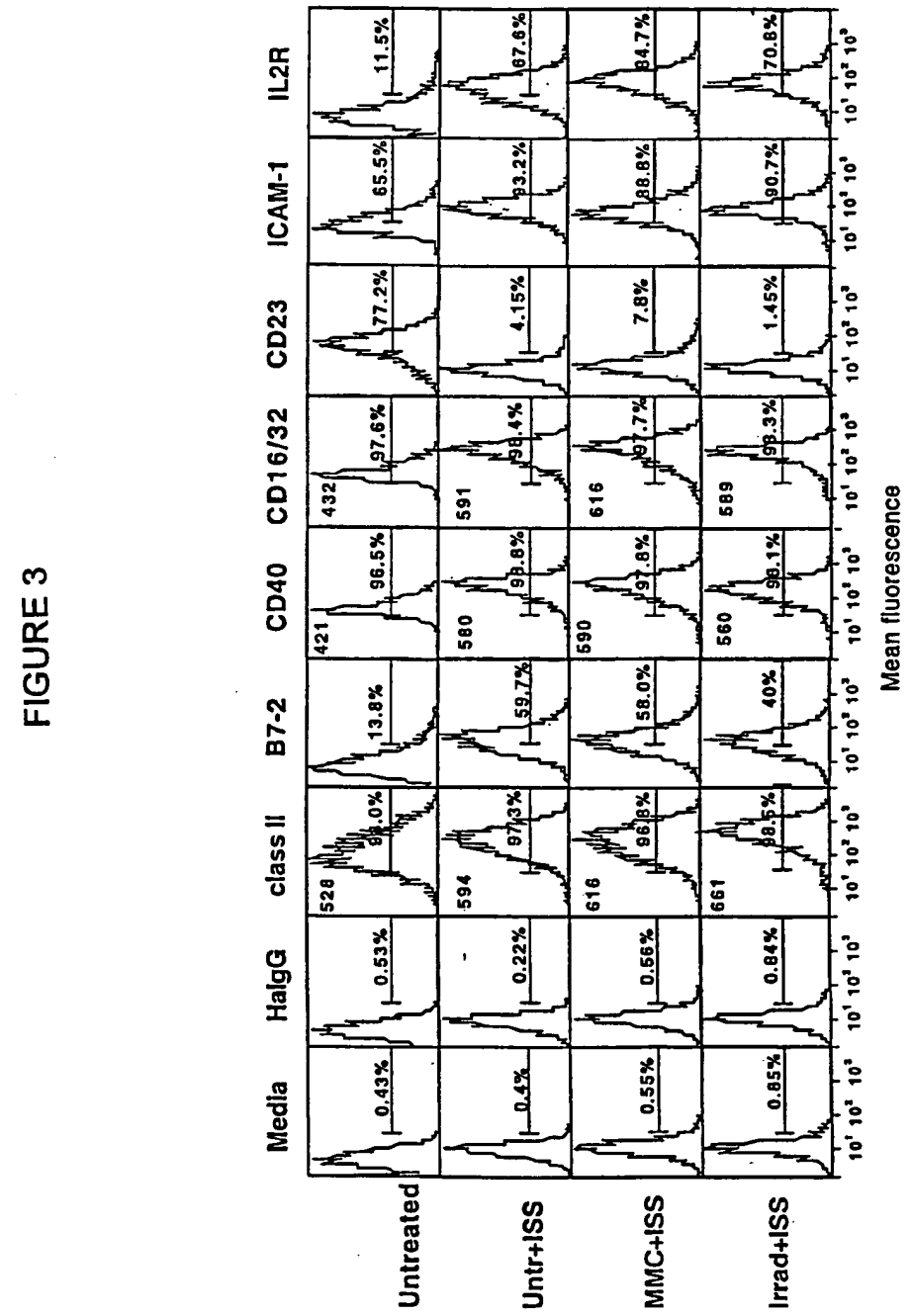

Media
HalgG
class II
B7-2
CD40
CD16/32
CD23
ICAM-1
IL2R
Untreated
Untr+ISS
MMC+ISS
Irrad+ISS
0.43%
0.53%
528
13.8%
421
96.5%
432
97.6%
77.2%
65.5%
11.5%
0.4%
0.22%
594
59.7%
580
98.8%
591
98.4%
4.15%
93.2%
67.8%
0.55%
0.56%
616
58.0%
590
97.8%
616
97.7%
7.8%
88.8%
84.7%
0.85%
0.84%
661
40%
560
98.1%
589
99.3%
1.45%
90.7%
70.8%
Mean fluorescence

FIGURE 4A

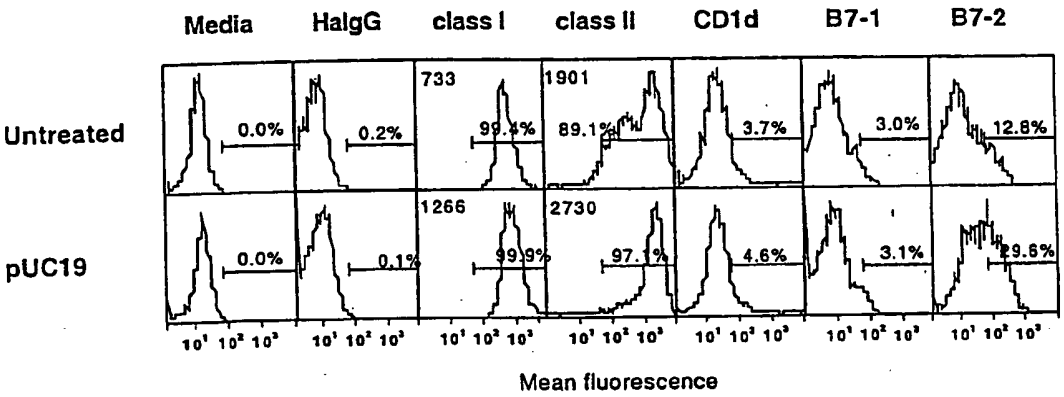


FIGURE 4B

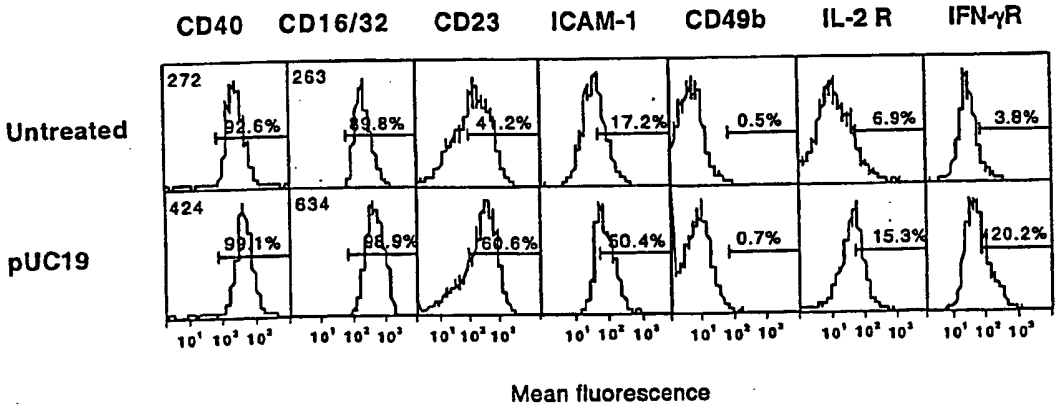


FIGURE 5A

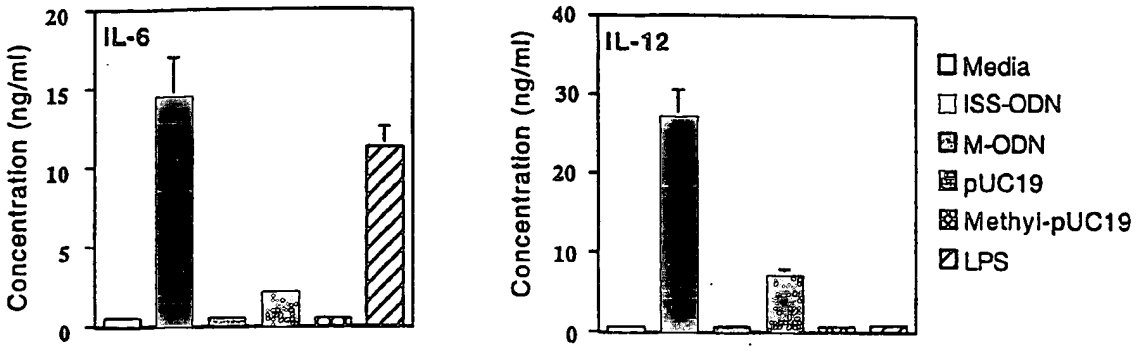


FIGURE 5B

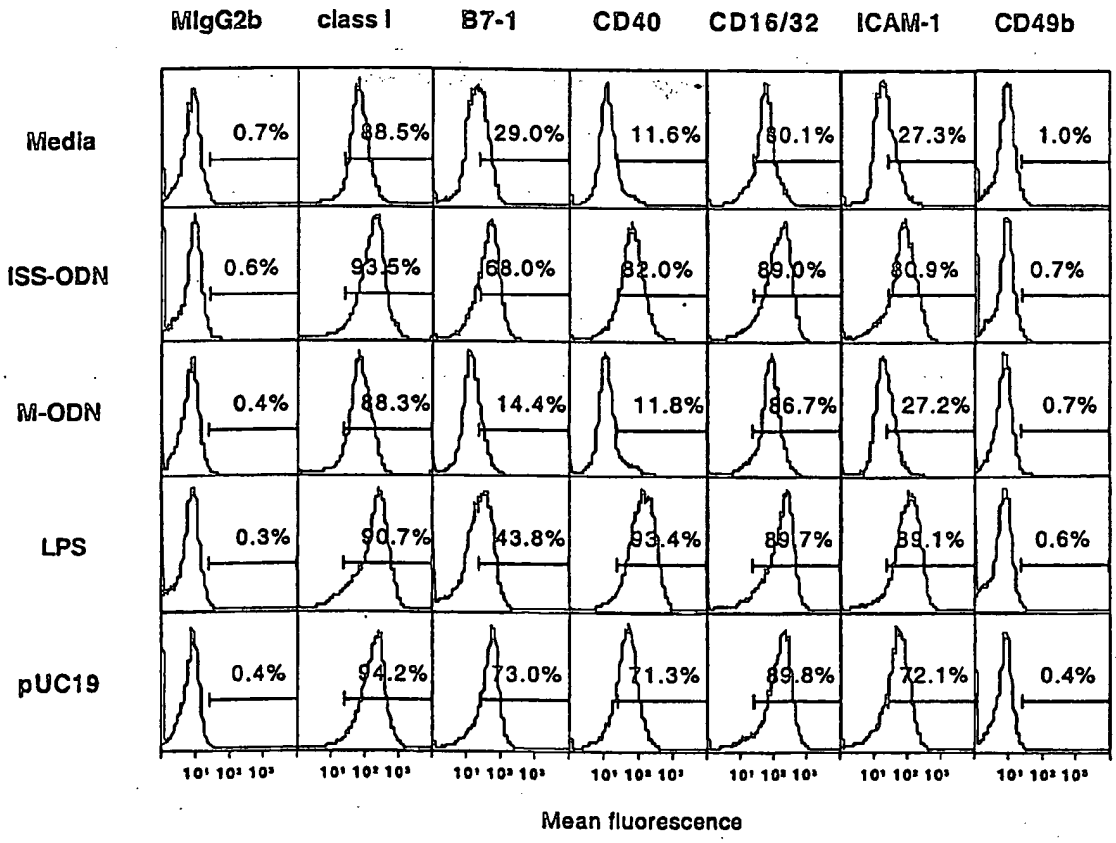


FIGURE 6A

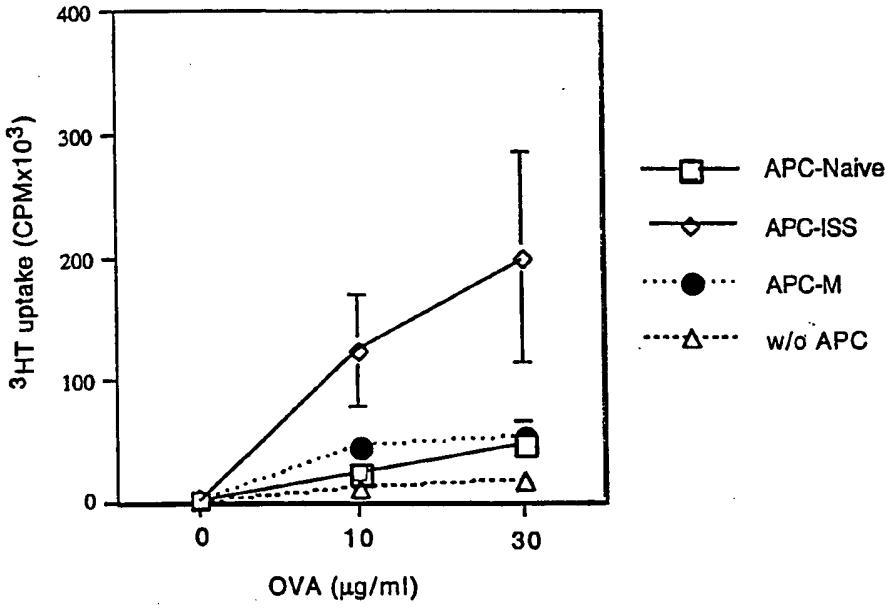


FIGURE 6B

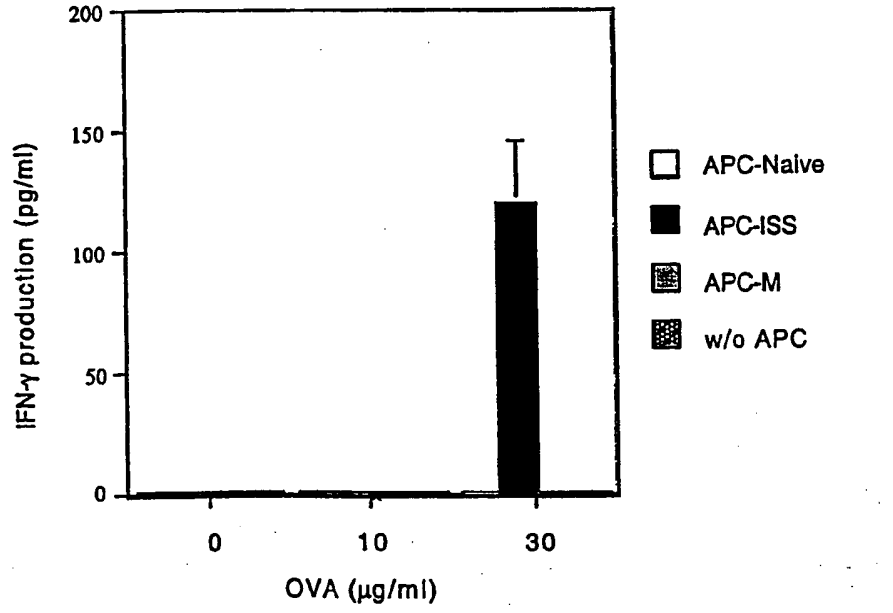


FIGURE 7A
IFN-γ Production

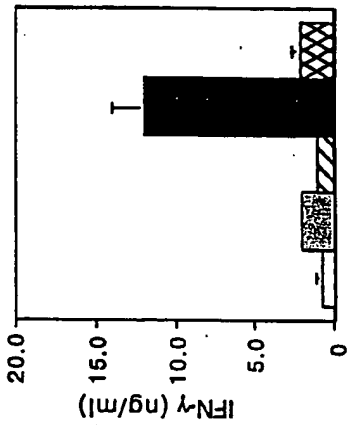
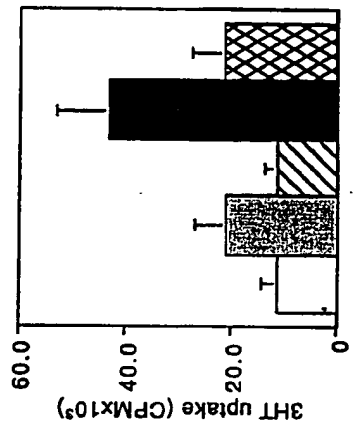
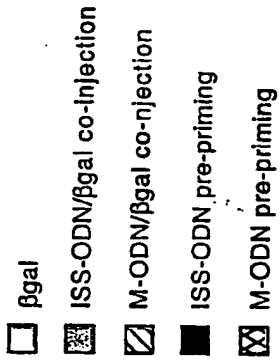


FIGURE 7B
T cell proliferation





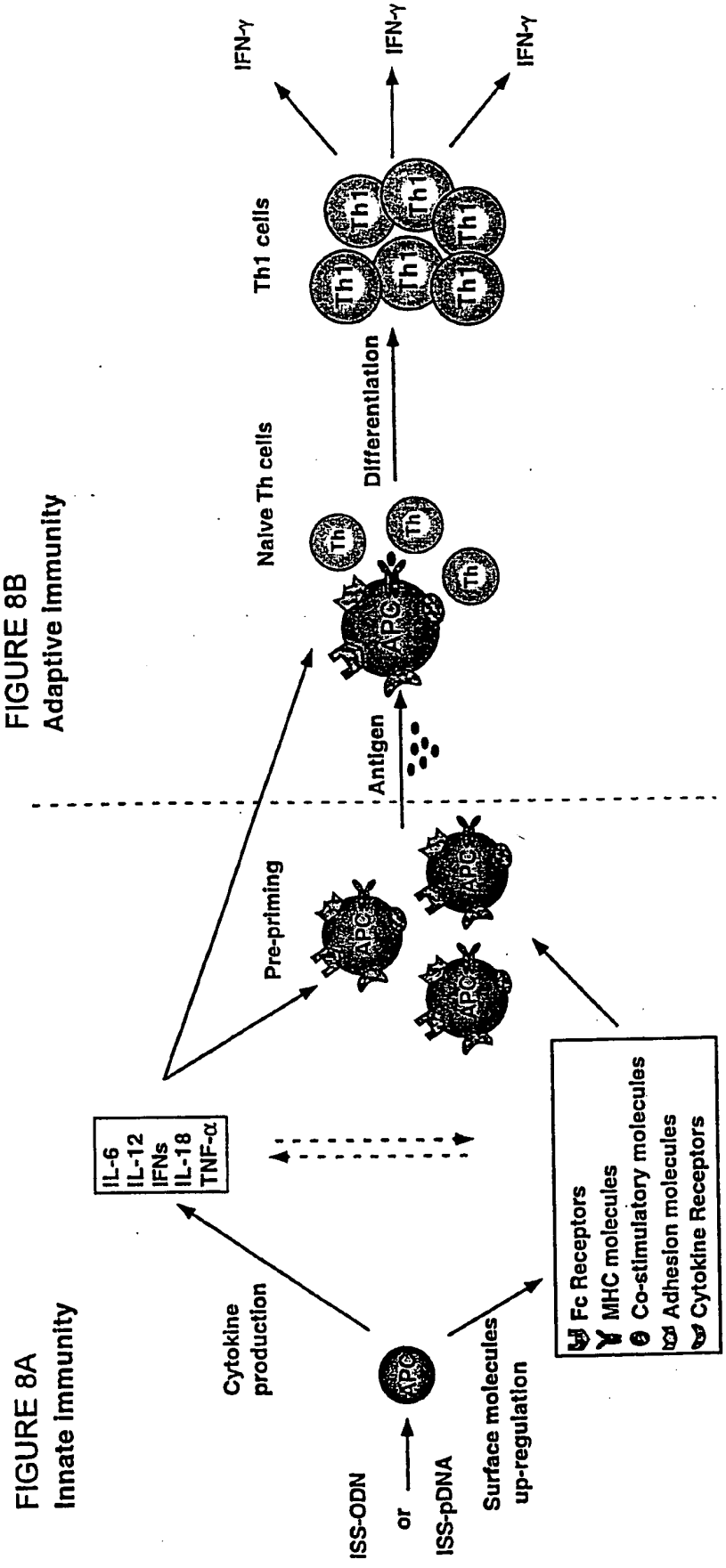


FIGURE 8A
Innate Immunity
ISS-ODN
or
ISS-pDNA
APC
Cytokine production
IL-6
IL-12
IFNs
IL-18
TNF-α
Surface molecules up-regulation
Fc Receptors
MHC molecules
Co-stimulatory molecules
Adhesion molecules
Cytokine Receptors
FIGURE 8B
Adaptive Immunity
Pre-priming
Antigen
Naive Th cells
Th
Differentiation
Th1 cells
Th1
IFN-γ

SEQUENCE LISTING

<110> RAZ, Eyal
MARTIN-OROZCO, Elena

<120> METHODS AND COMPOSITIONS FOR USE IN POTENTIATING ANTIGEN PRESENTATION BY ANTIGEN PRESENTING CELLS

<130> 06510/174WO1

<140> US 09/292,278
<141> 1999-04-15

<160> 18

<170> PatentIn Ver. 2.0

<210> 1
<211> 6
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Hexamer

<400> 1
aacgtt 6

<210> 2
<211> 6
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Hexamer

<400> 2
agcgtc 6

<210> 3
<211> 6
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Hexamer

<400> 3
gacgtt 6

<210> 4
<211> 6
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Hexamer

<400> 4
ggcgtt 6

<210> 5

<211> 6
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Hexamer

<400> 5
aacgtc 6

<210> 6
<211> 6
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Hexamer

<400> 6
agcgtc 6

<210> 7
<211> 6
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Hexamer

<400> 7
gacgtc 6

<210> 8
<211> 6
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Hexamer

<400> 8
ggcgtc 6

<210> 9
<211> 6
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Hexamer

<400> 9
aacgcc 6

<210> 10
<211> 6
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Hexamer

<400> 10

agcgcc 6

<210> 11
<211> 6
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Hexamer

<400> 11
gacgcc 6

<210> 12
<211> 6
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Hexamer

<400> 12
ggcgcc 6

<210> 13
<211> 6
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Hexamer

<400> 13
agcgct 6

<210> 14
<211> 6
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Hexamer

<400> 14
gacgct 6

<210> 15
<211> 6
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Hexamer

<400> 15
ggcgct 6

<210> 16
<211> 6
<212> DNA
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:Hexamer

<400> 16 6
ttcgaa

<210> 17
<211> 6
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Hexamer

<400> 17 6
ggcgtt

<210> 18
<211> 6
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Hexamer

<400> 18 6
aacgcc

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/09664

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : A61K 35/00, 48/00; C12N 15/63, 15/85, 15/86; A01N 1/02
US CL :514/44; 435/320.1, 325, 1.1; 424/93.1, 93.21

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/44; 435/320.1, 325, 1.1; 424/93.1, 93.21

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

NONE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	LIPFORD et al. Immunostimulatory DNA: sequence-dependent production of potentially harmful or useful cytokines. Dec. 1997. Eur. J. Immunol. Vol. 27, pages 3420-3426. See page 3425, column 1, line 2 and page 3422, sentence bridging columns 1 and 2.	1, 6, 12, 14-17, 20 and 21 --- 1-4 and 6-21
Y	JAKOB et al. Activation of cutaneous dendritic cells by CpG-containing oliodeoxynucleotides: a role for dendritic cells in the augmentation of Th1 responses by immunostimulatory DNA. J. Immunol. Vol. 161. pages 30342-3049. See page 3047, column 1, lind 5; 3048, sentence bridging columns 1 and 2.	1-4 and 6-21

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 MAY 2000	13 JUN 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer USPTO Telephone No. (703) 308-0000

Form PCT/ISA/210 (second sheet) (July 1998)☆

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/09664

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WOOLDRIDGE et al. Immunostimulatory oligodeoxynucleotides containing CpG motifs enhance the efficacy of monoclonal antibody therapy of lymphoma. Blood. April 15, 1997. Vol. 89. No. 8. page 2294-2998. See page 2995, column 1, last paragraph.	1 and 5